Characterization and Identification of dye degrading bacterial species from textile effluent

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IN
MICROBIOLOGY

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CERTIFICATE

This is to certify that the Internship Report entitled, **"CHARACTERIZATION AND IDENTIFICATION OF DYE DEGRADING BACTERIAL SPECIES FROM TEXTILE EFFLUENT"** submitted by **RUBY BISWAS (REG. NO.: 230010127775)** student of Department of Microbiology, Sister Nivedita University has successfully completed the Internship program from **02/01/2025** to **30/05/2025** in the Department of Life Sciences, **Presidency University, Kolkata** under the supervision of **Dr. Susmita Mondal**. She has worked independently under my guidance and completed the internship to my satisfaction.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "Characterization and Identification of dye degrading bacterial species from textile effluent" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from 2nd January, 2025 to 30th May, 2025 under the supervision of Dr. Susmita Mondal, Associate Professor, Department of Life Sciences, Presidency University, 86/1 College Street, Kolkata: 700073, West Bengal, India.

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TO WHOM IT MAY CONCERN

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Date: 5th June, 2025

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LIST OF ABBREVIATIONS

1. Indigo Carmine (I.C.)
2. Chemical Oxygen Demand (COD)
3. Biological Oxygen Demand (BOD)
4. Union Ministry of Environment and Forest (MoEF)
5. Total Suspended Solids (TDS)
6. Nicotinamide adenine dinucleotide (NADH)
7. Flavin adenine dinucleotide (FADH)
8. Luria Broth (L.B.)
9. Ultraviolet (UV) light
10. Pounds per square inch (psi)
11. Revolutions per minute (rpm)
12. 16S ribosomal RNA (16S rRNA)
13. Basic Local Alignment Search Tool (BLAST)
14. National Center for Biotechnology Information (NCBI)
15. Optical Density (O.D.)
16. Distilled water (d.H2O)

ABSTRACT

The rapid expansion of the textile industry has led to the discharge of significant amounts of dye-containing effluents into aquatic ecosystems, posing serious environmental and health hazards due to the toxic, carcinogenic, and persistent nature of synthetic dyes. Traditional physical and chemical methods for dye removal are often cost-intensive and generate secondary pollution. In this context, biological approaches, particularly microbial degradation, have emerged as promising eco-friendly alternatives. Denim effluent samples were collected from nearby denim industry wastewater sources and the 8 different bacterial strains were isolated and purified. Serial dilution and enrichment culture techniques were employed to isolate dye-tolerant bacteria using nutrient media supplemented with commonly used denim dye, i.e., Indigo Carmine. A total of 8 morphologically distinct bacterial isolates were obtained and screened for their dye-degradation efficiency under controlled laboratory conditions. The most efficient isolates demonstrated significant decolorization (above 70%) within 24 to 48 hours under aerobic conditions. These isolates were subjected to further biochemical characterization using standard microbiological techniques, including solid agar media plate dye degradation analysis, Luria broth (L.B.) dye degradation analysis, optical density (O.D.) measurement, etc. The dye degradation efficiency is significantly affected by the different parameters like pH, temperature, dye concentration, and incubation time. The findings suggest that indigenous bacterial species from textile effluents possess considerable potential for application in sustainable dye bioremediation processes. This study focuses on the characterization and identification of dye-degrading bacterial species isolated from textile effluents, with the aim of evaluating their potential for bioremediation. The effectiveness of microbial communities in the degradation of textile dyes underscores the importance of exploiting native bacterial strains for the development of cost-effective and environmentally benign wastewater treatment strategies. Further research into consortium-based approaches and pilot-scale applications could enhance the scalability and practical implementation of this biotechnological solution.

Keywords – textile industry, denim effluents, synthetic dyes, Indigo Carmine, dye-degradation, bacterial strains, bioremediation

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1. Introduction

Among all the textile industries, denim industries have gained significant popularity. A huge population of consumers have included denim jeans into their day-to-day fashion, and it has given rise to the increased production of denim. This has economically and globally made denim jeans to be the most used product that simultaneously increased the establishment of denim industries worldwide. These garments have proved to be comfortable, fit and stylish and that eventually has increased their production and manufacturing rate. Globally the production of denim jeans is reported to be 1.7 billion jeans every year and interestingly 70% of denim jeans factories are situated and functional in the Asian countries [1].Mostly the populous countries like China, India, Bangladesh are reported to contribute to the water pollution due to denim industries. 20% of the world's water pollution is due to the highly demanded production of textiles as stated by Greenpeace International [2]. Among the textile industries, denim industries release 45 to 65 liters of effluents per kilograms of denim [3]. Initially before 1856 in the textile industries, the dyes were prepared by natural sources, using flowers, vegetables, roots, woods, etc. From the end of 19th century, till now Indigo is prepared synthetically by the chemical processes and has been proved to be profitable chemical products in the textile industries to dye [4]. As the years passed the needs and demands of humans expanded and that gave rise to the synthetic dyes mainly manufactured by petrochemical sources. These synthetic dyes have advantages over the natural dyes, as the former was easily soluble in water, absorbed well and provided versatile colors with color fastness ability to the clothing [5,6]. There are different synthetic dyes used to dye denim fabrics and the degradation of each depends on the complexity of their chemical structures. Dyes when classified based on their chemical composition they are categorized as acid dyes, basic dyes, azo dyes, sulfur dyes, etc [6]. Among all dyes, Indigo Carmine (I.C.) is mostly used to dye denim garments because it imparts a characteristic blue color. During the process of dyeing the denim garments, indigo dye and cellulose in denim fabric creates a hydrogen bonding which is a new intermolecular force that is not stable and so due to repeated wearing & washing denim fades away. Indigo dye is a type of reducing dye that has a highly stable molecular structure so destroying its chromophore group by traditional oxidation and reduction techniques are not enough. It in turn damages the cotton fabric itself [7]. Indigo carmine dye has a complex chemical structure that has two important groups, $NaSO_3$ (that gives the ability to Indigo carmine to dissolve in water) and a chromophore group (gives the dye its characteristic blue color). The chromophore group

consisting of conjugated C=C bond is replaced by two C=O bond and two N-H groups [8]. Indigo carmine when dumped without treating the effluent, hazardously affects the aquatic ecosystem and health of human beings. It contaminates the water bodies by increasing the chemical oxygen demand (COD), increasing turbidity, stopping the sunlight to penetrate deep inside the water, affects plant photosynthesis and bacterial growth [8]. It has been estimated that around 2,00,000 tons of wastewater gets released from the textile industries due to the activities that are greatly water-intensive like dyeing, bleaching, washing and finishing activities. These wastewaters when dumped in the water bodies, change the natural biology and chemistry of the water by affecting water quality like its color, pH, biological oxygen demand (BOD), COD, total nitrogen, total solids, etc [9–11]. All the poisonous dyes from textile industries get released into the natural ecosystem like lakes, streams, ponds, and soils that contaminate each one of them. Due to the introduction of organic and inorganic toxins in the water bodies also become a great habitat for the pathogenic microorganisms to feed and survive that makes the wastewater more poisonous and threatens humans and animals that by any chance encounter wastewater [12,13]. When the dye gets accumulated in aquatic animals it can also inebriate the animals and humans by entering the food cycle [14]. Presence of even very low concentration of indigo dye makes the water non-potable, releases bad odor and changes watercolor [15]. These denim industries release the effluents that hazardously affect both on the human health and agricultural sector [2]. In every step of denim processing and dyeing toxic chemicals, auxiliaries and dyes are used. When they are dumped in an untreated manner contributes to the addition of hazardous contaminants in the water bodies. The dyes and chemicals used contain toxic heavy metals such as lead (Pb), chromium (Cr), cadmium (Cd), etc. can cause hazardous health effects like multiple organ failure affecting kidneys and nervous system also damaging skin, vascular and immune systems [2,16]. The heavy metals and metalloids released as effluent from textile industries into wastewater have great mutagenic and cancer-causing effects that totally disrupts the ecological imbalance [17,18]. Furthermore, these dyes in wastewater also prevent the sunlight from penetrating deep inside the dyed contaminated water bodies that decrease the availability of dissolved oxygen that is very significantly needed by the aquatic plants and animals for their growth and development. They have hazardous effect on the zooplanktons, phytoplanktons, amphibians, etc [19]. Therefore, it becomes very significant to treat the textile effluents with the remediation techniques that can decrease the water pollution and won't become a hazard for the environment. It will prevent the ecological balance from being disrupted which in turn will balance the economic and ecological parameters.

2. Review of Literature

The Industrial Revolution introduced the transformation of synthetic dye usage from natural dye in the textile industries. This transformation resulted in the rise of synthetic dyes to dye garments, being denim industries where denim garments are dyed mainly with synthetic ones. The denim industry uses several techniques in the denim processing and production, like removing impurities by chemical processes, dyeing, using chemical mordants to hold the dyes and finally finishing the manufacturing process. This results in using huge amounts of water and energy throughout the denim garments' production process and release of untreated effluents in the water bodies contaminating it hazardously [2]. A yearly report by the Union Ministry of Environment and Forest (MoEF) showed that around 13,011 industries or industrial units have been recorded to release approximately 4.4 million tons of the hazardous and toxic dyes into the nearby waterbodies in greater than 373 districts of a nation [20]. In everyday life these textiles industries have been recorded to release tons of wastewater due to the denim dyeing processes. An estimation showed that at the end of the nineteenth century when synthetic dye usage overpowered the natural dyes, approximately 10,000 synthetic dyes were produced, found and utilized in the industries for different type of techniques involved. The textile industries consume huge amounts of water for the different processes, specially dyeing which is the root point of releasing of different types of chemicals, auxiliaries and toxic dyes that when dumped in the water sources causes the clean water to be contaminated continuously [21]. In the dyeing process of blue denim, synthetic indigo dye is used commonly as a primary colorant to give denim garments it's worn-out appearance though indigo dyes are said to be poor quality vat dyes. It has been reported that the denim industries use 50,000 tons of indigo dye to give the denim its characteristic blue color. Synthetic indigo dyes are prepared chemically by the oxidation-reduction process [22]. Around 84,500 tons of sodium hydrosulphite and 53,500 tons of caustic soda are used each year [23,24]. These alkalis are used to convert the water insoluble indigo into soluble one and make it easy for the denim garments to dye them with that synthetic indigo [2,25]. The effluents in wastewater have hazardous characteristics like high pH, chemical oxygen demand (COD, from 340 to 17,900 mg/L), biological oxygen demand (BOD, from 210 to 5500 mg/L), total suspended solids (TDS, from 50 to 24,000 mg/L), and different colors from 300 to 3500 color units, etc. that contaminates the water and causes ecological imbalance. These abnormalities increase turbidity in the water bodies and prevent the sunlight from entering it [26,27]. One of the biggest drawbacks here is that the indigo dye doesn't totally get fixed on the denim fabric, but some remain unfixed and during

the washing process these unfixed dyes are washed out as effluents and dumped as wastewater. This is a threat to the environment as the water gets highly turbid and the ecological imbalance is caused by the wastewater poisonous, carcinogenic and mutagenic [2,28]. This results as a threat to the environment and nearby residents who can easily come om contact with these contaminated wastewaters. Due to this type of problems, it is very significant to treat the wastewater well enough before releasing them into the waterbodies that can lower the contamination and water pollution level and won't become a threat to the environment. So, various physico-chemical techniques are used to remediate the industrial effluents before directly releasing them into the water sources [29]. The source of water used for irrigation in agricultural fields and the dumping of wastewater into the various water sources are the same, therefore it also becomes a major concern for treating the industrial effluents before being released as the effluents contain wide range of toxins and hazardous chemicals that cause ecological imbalance. Additionally, the dye particles introduced into the atmosphere also combines with the rain and flows into the water bodies [30]. When water from contaminated sources is used for irrigation purposes in the agricultural fields then they affect the necessary microbial communities of soil, in turn affecting the germination, growth and development of the plants [31]. Dye containing effluents are reported to be hazardous because they contain toxic chemicals such as organic & inorganic compounds, pigments, xenobiotics, alkali salts, formaldehyde, chlorine, aromatic amines and heavy metals. Therefore, this shows detrimental effects in the soil ecology and brings about both soil and water pollution [32,33]. These textile dyes are recalcitrant and toxic that causes bioaccumulation, hampers the photosynthesis in plants, inhibits plant growth and enters the food chain because plants are consumed in context of agriculture thereby inducing carcinogenicity, mutagenicity and toxicity [34]. Denim dye degradation is therefore a main concern in today's time to save the future ecological balance from further damage caused due to the toxic effects of untreated denim dyes directly getting dumped into water bodies. Various physical and chemical remediation techniques have been involved for removing the dyes and still denim garments their worn-out appearance, softness and easy handling. The processes include oxidizing-bleaching treatment [35], stonewashing [36], electrochemical reduction [37], bio-washing, laser and plasma treatment, enzymatic treatment and fusion of nanoparticles with enzymes [38]. Oxidizing-bleaching treatment and stonewashing have shown negative impacts on the finished denim garments and so later electrochemical reduction was considered to use to bleach denim fabrics by producing radicals but wasn't too efficient enough [39]. Enzymatic treatment has also been reported to be another environment friendly technique using different enzymes like pure laccase [40], pure acid cellulase [41], pure

neutral cellulase [42], mixture of liquid ammonia/cellulase [43], mixture of cellulase/methacrylate copolymer [44], etc. Plasma and laser treatment introduced as efficient techniques are environmentally friendly as they decrease the use of huge amounts of water and chemical agents in textile industries. Although, safety measures must be kept in mind regarding the laser treatment because they can damage the denim structure and reduce their tensile strength [45]. Nanoparticles and reducing agents together are also used to degrade the indigo dye by reducing silver nitrate in the presence of glucose/starch on the denim garments, where glucose reduces indigo dye. Besides, antibacterial activity on the denim was also observed against *Escherichia coli* and *Staphylococcus aureus* [46,47]. These physico-chemical techniques have given good wastewater remediation results but still they are considered environmentally toxic because these techniques involve the usage of various chemical agents and fusion of different chemical agents, synthetic substances and salts. These remediate the wastewater but as considerable side effects give rise to secondary pollutants that in turn increases the water pollution level [48,49]. Therefore, limiting the use of physical and chemical remediation, bioremediation techniques are used greatly. Bioremediation have proved to be environmentally friendly, cost-effective and more efficient than other remediation techniques by using the plants and microbial species due to their non-toxic nature. As the wastewater remediation becomes a part of plant and microbial species naturally and metabolically, it decreases the minimal use of techniques for treating the industrial effluents [50]. Bioremediation techniques are more efficient compared to physical and chemical pretreatments [51]. Traditional biological processes are cost-effective and ecologically sustainable techniques that reduces the effluent production by using few chemicals, saving energy and being environmentally friendly unlike physico-chemical remediation process [52]. Bioremediation of dyes can be achieved by using microorganisms like bacteria, fungi, algae, and yeast [53].These organisms' function differently based on their degradation efficiency, limitations and working conditions like pH, carbon source, temperature, dye concentration [54]. The process of dye degradation occurs mainly in two stages in both aerobic and anaerobic conditions [55]. Due to the low redox potential, it has been observed that the dye degradation by bacteria occurs more efficiently in anaerobic conditions [56]. Bacterial dye degradation under aerobic condition is catalysed by enzymes, majorly azo reductases that reduces azo dyes and releases intermediate metabolites like aromatic amines that again goes under final degradation in aerobic condition. In anaerobic condition, azo reductases break azo bonds of the dye releasing the redox intermediates like NADH/FADH and decolorization of dye takes place in two steps by transfer of four electrons[56] . Variety of fungal and bacterial strains have been reported to perform successful denim dye

biodegradation. Fungi such as *Aspergillus sp., Penicillium sp., and Hypocrea koningii* have shown the dye degradation capability [57,58]. Some bacterial strains also have the decolorization capability of various industrial dyes. They are *Enterococcus faecalis*, *Bacillus spp.*, *Clostridium butyricum*, *Klebsiella oxytoca* etc [59]. Experimentally it was seen that nineteen isolates among thirty-five showed the indigo dye decolourizing activities. The isolates were identified as *Bacillus* BT5, *Bacillus* BT9, *Lactobacillus* BT20, and *Lysinibacillus* BT32 and a fungal isolate *Aspergillus* H1T. *Lactobacillus* BT20 showed 90% indigo dye decolorization and glucose consumption within 264 hours of incubation in aerobic conditions. In absence of glucose its decolorization capability decreased drastically [60,61]. Enzyme called azoreductases in the *Lactobacillus* species showed the degradation of azo dyes like acid black under aerobic conditions [62]. Bacillus BT9 showed 59% decolorization ability in absence of glucose by modifying the blue-coloured dye into red-coloured metabolites. The enzyme called Laccase is responsible for the decolorization effect [63,64]. Reportedly *Bacillus* species have also shown indigo carmine decolorization and then forming indoline sulfonic acid [65]. *Bacillus* BT5 and *Lysinibacillus* BT32 have shown 30% to 36% indigo dye decolorization in absence of glucose. *Aspergillus* H1T also showed 96% of decolorization within 2 days by forming blue-coloured spherical masses of mycelium. Lignin-peroxidases and laccases enzymes are present in *Aspergillus* species that degrades the dye [66,67]. Some recent advancements and modifications in the bioremediation techniques have been reported to provide better results technically, effectively, environmentally friendly and feasibly [68]. The advancements for wastewater bioremediation are notably nanotechnology and its advancements, microbial electrochemical technologies (MET), strain improvement for enzyme immobilization, genetic engineering and phytoremediation [52,69]. Bioremediation techniques are indeed the best to remediate the toxic textile effluents before releasing them into the nearby water bodies, but it also has some drawbacks. Environmental and nutritional elements can influence the dye bioremediation [70]. The study revealed that the bioremediation of the dyes is dependent on the dye's molecular structure, particular microbial species involved in bioremediation, biomass content and the physicochemical influences such as temperature and pH. All these parameters must be optimized to achieve biological remediation for the textile effluents. The limiting factors that can prove to be the significant drawbacks in utilizing the bioremediation to treat the effluents are lower pH, higher temperature, and in many cases anaerobic conditions. Due to these sensitive optimization characteristics bioremediation isn't used so popularly by the textile industries to treat the effluents that give rise to great amount of pollution [71].

3. Materials and methods

3.1 Apparatus and materials required

1. Weighing balance
2. 96 microtiters well plate reader
3. Conical flask
4. Beaker
5. Spatula
6. Measuring cylinder
7. 90 mm petri plates
8. Test tubes and rack
9. Culture tubes
10. Centrifuge tubes and rack
11. Falcon tube containing I.C. dye
12. Inoculating loop
13. Toothpicks
14. 1ml syringe and needle
15. Pipette and pipette tips
16. Spirit lamp
17. Gloves and mask
18. Cotton plugs
19. Parafilm
20. Aluminium foil
21. Tissue
22. Scissor
23. Marker
24. Distilled water

3.2 Reagents required

1. Luria Broth powder
2. Agar powder
3. Indigo Carmine dye
4. 70% Ethanol

4. Methodology

4.1) Preparation of L.B. agar plates supplemented with I.C. dye (50 µg/ml) in 100ml L.B. agar media

250 ml of conical flask was taken. L.B. and agar powder were measured in the weighing balance and were taken in the conical flask. Then 100 ml of distilled water was measured using the measuring cylinder to pour into the conical flask to prepare 100 ml of Luria agar media.

Calculation for LB agar media preparation (Total volume – 100 ml): -

- LB powder – 20 gms in 1000 ml
  So, in 100 ml = 20/1000*100 = 2 gms
- Agar powder – 15 gms in 1000 ml
  So, in 100 ml = 15/1000*100 = 1.5 gms

After mixing the media well, the conical flask was cotton plugged and was given for autoclaving at 121º C for 15 minutes at 15 psi. After the autoclave was done, it was taken out of the autoclave. In the laminar air flow, all the required materials were exposed to the ultraviolet (UV) light for 30-45 minutes for sterilization. Then 50µg/ml of I.C. dye was measured and pipetted out from the 10 mg/ml of I.C. stock solution and added into the agar media.

Calculation for 50 µg/ml of I.C. dye (Total volume – 100 ml): -

- S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml
- S2 (Concentration to be achieved) = 50 µg/ml
- V1 (Volume to be added) = X
- V2 (Final volume) = 100 ml = 100*10^3 µl

Using the formula, V1*S1 = V2*S2;

V1 * S1 = V2 * S2

X * (10^3*10 µg/ml) = (100 *10^3 µl) * (50 µg/ml)

X = [(100*10^3 µl) * (50 µg/ml)] / [(10^3*10 µg/ml)]

X = 500 µl

Therefore, the volume of I.C. dye that was added (V1) into the agar media was calculated to be 500 µl. Two plates for quadrant streaking with the TB1 and TB2 strains and another

two plates for their control, respectively were exposed to the UV light for sterilization inside the laminar air flow. After adding the I.C. dye, the media and the dye were stirred and mixed manually, and they were poured carefully into the 4 petri plates. Then as the plates got solidified, they were ready for the quadrant streaking to be performed.

4.2) Quadrant streaking in L.B. agar plates supplemented with I.C. dye (50 µg/ml)

The 4 I.C. dye supplemented L.B. agar plates were exposed to the UV for 30-45 minutes inside the laminar air flow for sterilization. Glycerol stock solutions of TB 1 and TB 2 strain were taken out of -80º C freezer. Hands were sanitized by 70% ethanol, then sterilized gloves and masks were worn. Two plates were separately marked using the marker as TB1 plate and TB2 plate to avoid any confusion followed by marking other two plates as control for both TB1 and TB2. One sterilized toothpick was carefully taken out of the beaker and was dipped in the TB1 glycerol stock solution followed by streaking the first quadrant lines on the L.B. agar plates. For the rest of the 4 quadrants to be streaked 4 different sterilized toothpicks were used to draw the streak lines. Similarly, the quadrant streaking was performed for the other strain, TB2. After streaking all the 4 plates were carefully wrapped with parafilm, they were placed inside the incubator at 37º C for 24 hours of observation.

4.3) Preparation of L.B. solid agar media plates supplemented with different I.C. dye concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml) in 50ml L.B. agar media

4 conical flasks of 100ml were taken to prepare 50 ml LB agar media in each flask. The 4 different flasks were taken to prepare 4 different I.C. dye concentrated (50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml) L.B. agar media plates. The same amount of L.B. powder and agar powder were mixed in each conical flask by measuring and adding 50 ml of distilled water using the measuring cylinder.

Calculation for LB agar media preparation (Total volume – 50 ml): -

- LB powder – 20 gms in 1000 ml
  So, in 100 ml = 20/1000*50 = 1 gm
- Agar powder – 15 gms in 1000 ml
  So, in 100 ml = 15/1000*50 = 0.75 gms

After, all the conical flasks containing the LB agar media, were stirred and mixed well by adding 50 ml distilled water. Then all the flasks were plugged in cotton. Then they were autoclaved at 121º C for 15 minutes and 15 psi. After the autoclaving was done, the conical flasks were taken out and different I.C. dye concentrations were calculated and pipetted into each respective conical flask.

Calculation for 50 µg/ml of I.C. dye (Total volume – 50 ml): -

- S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml
- S2 (Conc. To be achieved) = 50 µg/ml
- V1 (Volume to be added) = X
- V2 (Final volume) = 50 ml = 50*10^3 µl

Using the formula, V1*S1 = V2*S2;

V1 * S1 = V2 * S2

X * (10^3*10 µg/ml) = (50 *10^3 µl) * (50 µg/ml)

X = [(50*10^3 µl) * (50 µg/ml)] / [(10^3*10 µg/ml)]

X = 250 µl

Calculation for 100 µg/ml of I.C. dye (Total volume – 50 ml): -

- S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml
- S2 (Concentration to be achieved) = 100 µg/ml
- V1 (Volume to be added) = X
- V2 (Final volume) = 50 ml = 50*10^3 µl

Using the formula, V1*S1 = V2*S2;

V1 * S1 = V2 * S2

X * (10^3*10 µg/ml) = (50 *10^3 µl) * (100 µg/ml)

X = [(50*10^3 µl) * (100 µg/ml)] / [(10^3*10 µg/ml)]

X = 500 µl

Calculation for 200 µg/ml of I.C. dye (Total volume – 50 ml): -

- S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml
- S2 (Concentration to be achieved) = 200 µg/ml

- V1 (Volume to be added) = X
- V2 (Final volume) = 50 ml = $50*10^3$ µl

Using the formula, V1*S1 = V2*S2;

V1 * S1 = V2 * S2

X * (10^3*10 µg/ml) = (50 *10^3 µl) * (200 µg/ml)

X = [(50*10^3 µl) * (200 µg/ml)] / [(10^3*10 µg/ml)]

X = 1000 µl (1ml)

Calculation for 300 µg/ml of I.C. dye (Total volume – 50 ml): -

- S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml
- S2 (Concentration to be achieved) = 300 µg/ml
- V1 (Volume to be added) = X
- V2 (Final volume) = 50 ml = $50*10^3$ µl

Using the formula, V1*S1 = V2*S2;

V1 * S1 = V2 * S2

X * (10^3*10 µg/ml) = (50 *10^3 µl) * (300 µg/ml)

X = [(50*10^3 µl) * (300 µg/ml)] / [(10^3*10 µg/ml)]

X = 1500 µl (1.5 ml)

Therefore, each of the conical flasks were supplemented with each different I.C. dye concentrations. The 20-200 µl pipette and tips was used to pipette 250 µl of I.C. dye into one conical flask having the 50 µg/ml dye concentration, 500 µl of I.C. dye was pipetted into second conical flask having the 100 µg/ml dye concentration, 1000 µl of I.C. dye was pipetted into third conical flask having 200 µg/ml and 1500 µl of I.C. dye was pipetted into the last conical flask having 300 µg/ml dye concentration. After this all the 4 conical flasks were stirred to mix the dye with the L.B. agar media. Then from each conical flask, media was poured into two petri plates, one plate to be used for quadrant streaking and the other one was its control plate. In this was a total of 8 plates that were plated for both strains, TB1 and TB2. After the plates were solidified, they were ready to be streaked upon.

4.4) Quadrant streaking of TB1 and TB2 bacterial species on 50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml LB + I.C. dye concentrated agar media plates

In the same way for both the strains of bacteria (TB1 and TB2), the quadrant streaking was performed in 4 different L.B. agar media plates supplemented with 4 different I.C. dye concentration. All the 8 plates were exposed to the UV light inside the laminar airflow for 30-45 minutes for sterilization. Then hands were sanitized with 70% ethanol, followed by the gloves and mask carefully put on. With the help of one sterilized toothpick the streak lines on the first quadrant were done followed by each toothpick for the rest of the 3 quadrants. After completing the quadrant streak on the 4 plates with 50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml dye concentration, leaving the other 4 as their respective control plates, all the 8 plates were carefully wrapped with the parafilm. The plates were then kept inside the incubator at 37º C for 24 hours to observe the dye degradation capacity of both TB1 and TB2, respectively. After 24 hours observation, the plates were again kept in the incubator for the next 24 hours, total 48 hours to observe more dye degradation phenomenon by both the bacterial strains, TB1 and TB2.

4.5) Preparation of Luria broth solution supplemented with different I.C. dye concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml) in 10ml of LB

A total of 8 culture tubes were taken, and L.B. powder was measured in the weighing balance and added into the tubes. Then in each tube 10 ml of distilled water was measured using the 100-1000 µl pipette and tips and was pipetted into the culture tubes separately and respectively.

Calculation for Luria Broth preparation (Total volume – 10 ml): -

- LB powder – 20 gms in 1000 ml
  So, in 10 ml = 20/1000*10 = 0.2 gms
  Therefore, 0.2 gms of Luria Broth powder was measured and added into each of the 8 culture tubes. Then 10 ml of distilled water was measured, and the L.B. media was mixed well by manually stirring the tubes. Then all the tubes were given for autoclaving at 121º C for 15 minutes and 15 psi. After the autoclave was done, the culture tubes were kept in the room temperature. Then in the 8 culture tubes different concentrations of I.C. dye was calculated to supplement the Luria broth.

Calculation for 50 µg/ml of I.C. dye to be added to Luria broth media (Total volume – 10 ml): -

S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml

S2 (Concentration to be achieved) = 50 µg/ml

V1 (Volume to be added) = X

V2 (Final volume) = 10 ml = 10*10^3 µl

Using the formula, V1*S1 = V2*S2;

V1 * S1 = V2 * S2

X * (10^3*10 µg/ml) = (10 *10^3 µl) * (50 µg/ml)

X = [(10*10^3 µl) * (50 µg/ml)] / [(10^3*10 µg/ml)]

X = 50 µl

Calculation for 100 µg/ml of I.C. dye to be added to Luria broth media (Total volume – 10 ml): -

S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml

S2 (Concentration to be achieved) = 100 µg/ml

V1 (Volume to be added) = X

V2 (Final volume) = 10 ml = 10*10^3 µl

Using the formula, V1*S1 = V2*S2;

V1 * S1 = V2 * S2

X * (10^3*10 µg/ml) = (10 *10^3 µl) * (100 µg/ml)

X = [(10*10^3 µl) * (100 µg/ml)] / [(10^3*10 µg/ml)]

X = 100 µl

Calculation for 200 µg/ml of I.C. dye to be added to Luria broth media (Total volume – 10 ml): -

S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml

S2 (Concentration to be achieved) = 200 µg/ml

V1 (Volume to be added) = X

V2 (Final volume) = 10 ml = 10*10^3 µl

Using the formula, V1*S1 = V2*S2;

$V1 * S1 = V2 * S2$

$X * (10^3*10\ \mu g/ml) = (10 *10^3\ \mu l) * (200\ \mu g/ml)$

$X = [(10*10^3\ \mu l) * (200\ \mu g/ml)] / [(10^3*10\ \mu g/ml)]$

$X = 200\ \mu l$

Calculation for 300 µg/ml of I.C. dye to be added to Luria broth media (Total volume – 10 ml): -

S1 (Stock solution) = 10x mg/ml = 10^3*10 µg/ml

S2 (Concentration to be achieved) = 300 µg/ml

V1 (Volume to be added) = X

V2 (Final volume) = 10 ml = $10*10^3$ µl

Using the formula, V1*S1 = V2*S2;

$V1 * S1 = V2 * S2$

$X * (10^3*10\ \mu g/ml) = (10 *10^3\ \mu l) * (300\ \mu g/ml)$

$X = [(10*10^3\ \mu l) * (300\ \mu g/ml)] / [(10^3*10\ \mu g/ml)]$

$X = 300\ \mu l$

After all the calculations, the different I.C. dye concentrations were known to be supplemented with the L.B. media prepared in the culture tubes. One set of culture tubes containing 4 tubes were supplemented separately by 4 different I.C. dye concentrations. Using the 20-200 µl pipette and tips, 50 µl I.C. dye was pipetted in two culture tubes, 100 µl I.C. dye was pipetted in another two culture tubes, 200 µl and 300 µl of I.C. dye was pipetted each into two culture tubes respectively to inoculate the TB1 and TB2 bacterial strains.

4.6) Inoculation of TB1 and TB2 bacterial species in L.B. + I.C. dye concentrations of 50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml in broth media prepared

All the 8 culture tubes with L.B. + different I.C. dye concentrations broth prepared were exposed to UV light inside the laminar airflow for 30-45 minutes for sterilization purposes. After the UV, hands were sanitized well with 70% ethanol followed by carefully wearing gloves and mask. The working station was sanitized by wiping it with 70% ethanol and then the streaked agar plates of TB1 and TB2 were taken out. The parafilm was unwrapped very carefully and by heating the inoculating loop until it became red hot that shows the

sterilization of the loop to prevent any possible cross – contamination between the two bacterial sample, TB1 and TB2. Then as the loop was cooled down, 1 inoculum of TB1 was taken carefully and inoculated one by one in all the 4 culture tubes containing 4 different I.C. dye concentrations + L.B. broth. The rest of the 4 culture tubes weren't inoculated with TB1 strain as they were control tubes for TB1 inoculated tubes. The same process was followed to inoculate the TB2 bacterial strain in the culture tubes. Then all the inoculated tubes with their respective control sets were kept for incubation at 37º C for 24 and 48 hours in static condition and in shaking condition at 160 rpm at 37º C for both 24 and 48 hours to observe and characterize the dye – degradation capacity.

5. Results and discussions

5.1) Revival of bacterial strains from glycerol stock solution

Previously the water sample was collected from the denim wastewater, in a container. By performing the serial dilution process, each of the segregated diluted samples were plated using the spread plate technique. The plates were incubated for 24 hours at 37ºC. From the spread plates, morphologically different kinds of bacterial strains were isolated, and they were further purified into 8 different bacterial strains namely, TB1, TB2, TB3, TB4, TB5, TB6, TB7 and TB8. All these isolated strains were then stored in the already prepared 10mg/ml of glycerol stock solution.

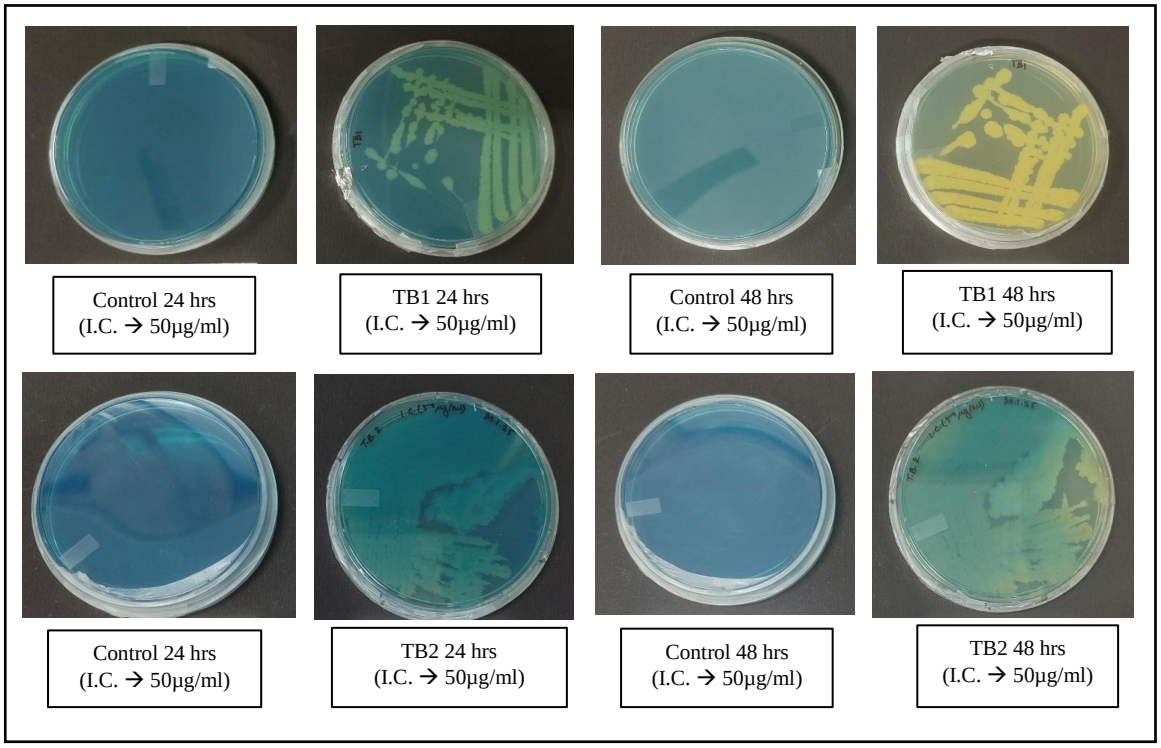


Figure 1: Revival of TB1 and TB2 isolated bacterial strains from glycerol stock solution after 24 and 48 hours of observation

Among all these strains, I had to work with two bacterial strains, TB1 and TB2. These two bacterial strains were revived from the glycerol stock solution and were streaked on the solid agar media plates by using the quadrant streaking method. Those plates were supplemented with 50 µg/ml Indigo Carmine (I.C.) dye concentration and kept on incubation at 37º C for 24 hours. After 24 hours, the plates were observed for the bacterial species growth that proved the bacterial strains, TB1 and TB 2 were successfully revived and were used further for the different dye–degradation analysis.

After this the plates were also kept again for 24 hours, i.e., in total 48 hours of incubation at 37° C. This was done to observe that both the bacterial strains have revived successfully and are showing total dye-degradation after 48 hours of incubation.

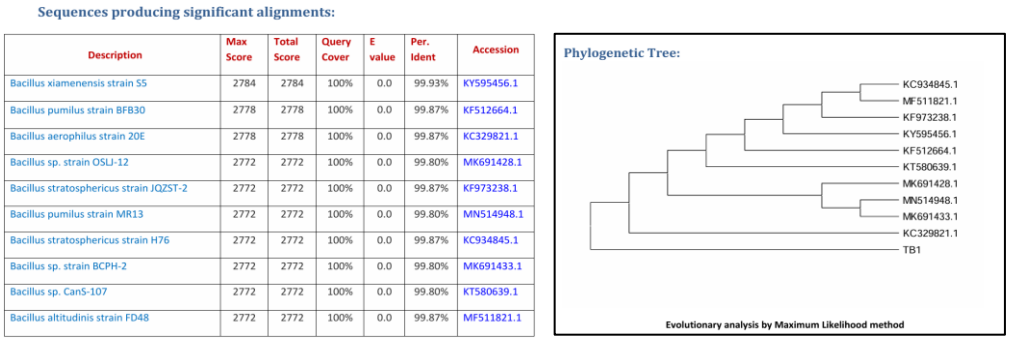
5.2) Colony morphology of the TB1 and TB2 bacterial species streaked on the LB agar media plates

Sl.No.	Sample Name	Colony Form	Colony Elevation	Colony Margin	Colony colour
1.	TB1 (*Bacillus xiamenensis*)	Circular	Raised	Entire	White
2.	TB2 (*Bacillus licheniformis*)	Regular	Raised	Lobate	White

Table 1: Colony morphology of the TB1 and TB2 bacterial species streaked on the LB agar media plates

TB1 and TB2 strains showed the different colony morphology when they were streaked on the L.B. agar plates. Both the strains were observed to be white in color. The colony formation of TB1 strain was observed to be circular, raised elevation and entire colony margin. TB2 showed the regular colony form with raised elevation and lobate colony margin. Some vesicular like structures were observed in the TB2 streaked plates containing some liquid substance.

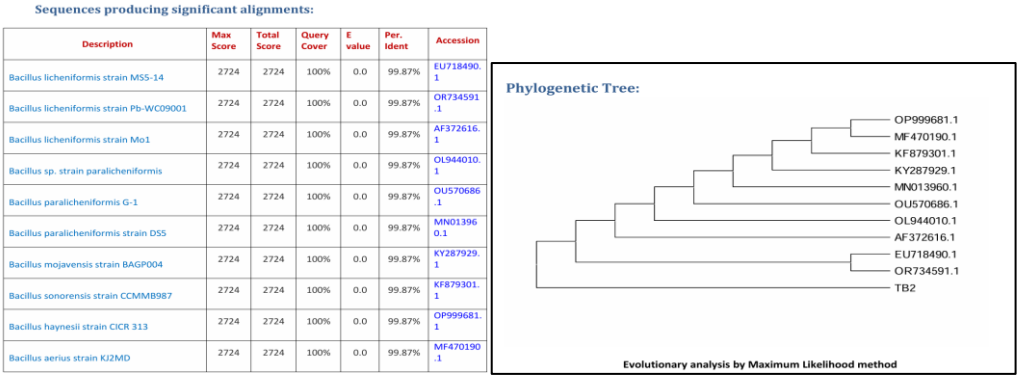
5.3) Identification of bacterial strains isolated from textile wastewater



Sequences producing significant alignments:

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus xiamenensis strain S5	2784	2784	100%	0.0	99.93%	KY595456.1
Bacillus pumilus strain BFB30	2778	2778	100%	0.0	99.87%	KF512664.1
Bacillus aerophilus strain 20E	2778	2778	100%	0.0	99.87%	KC329821.1
Bacillus sp. strain OSLJ-12	2772	2772	100%	0.0	99.80%	MK691428.1
Bacillus stratosphericus strain JQZST-2	2772	2772	100%	0.0	99.87%	KF973238.1
Bacillus pumilus strain MR13	2772	2772	100%	0.0	99.80%	MN514948.1
Bacillus stratosphericus strain H76	2772	2772	100%	0.0	99.87%	KC934845.1
Bacillus sp. strain BCPH-2	2772	2772	100%	0.0	99.80%	MK691433.1
Bacillus sp. CanS-107	2772	2772	100%	0.0	99.80%	KT580639.1
Bacillus altitudinis strain FD48	2772	2772	100%	0.0	99.87%	MF511821.1

Figure 2: Identification of TB1 via 16S rRNA sequencing – *Bacillus xiamenensis*



Sequences producing significant alignments:

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus licheniformis strain MSS-14	2724	2724	100%	0.0	99.87%	EU718490.1
Bacillus licheniformis strain Pb-WC09001	2724	2724	100%	0.0	99.87%	OR734591.1
Bacillus licheniformis strain Mo1	2724	2724	100%	0.0	99.87%	AF372616.1
Bacillus sp. strain paralicheniformis	2724	2724	100%	0.0	99.87%	OL944010.1
Bacillus paralicheniformis G-1	2724	2724	100%	0.0	99.87%	OU570686.1
Bacillus paralicheniformis strain DS5	2724	2724	100%	0.0	99.87%	MN013960.1
Bacillus mojavensis strain BAGP004	2724	2724	100%	0.0	99.87%	KY287929.1
Bacillus sonorensis strain CCMMB987	2724	2724	100%	0.0	99.87%	KF879301.1
Bacillus haynesii strain CICR 313	2724	2724	100%	0.0	99.87%	OP999681.1
Bacillus aerius strain KJ2MD	2724	2724	100%	0.0	99.87%	MF470190.1

Figure 3: Identification of TB2 via 16S rRNA sequencing – *Bacillus licheniformis*

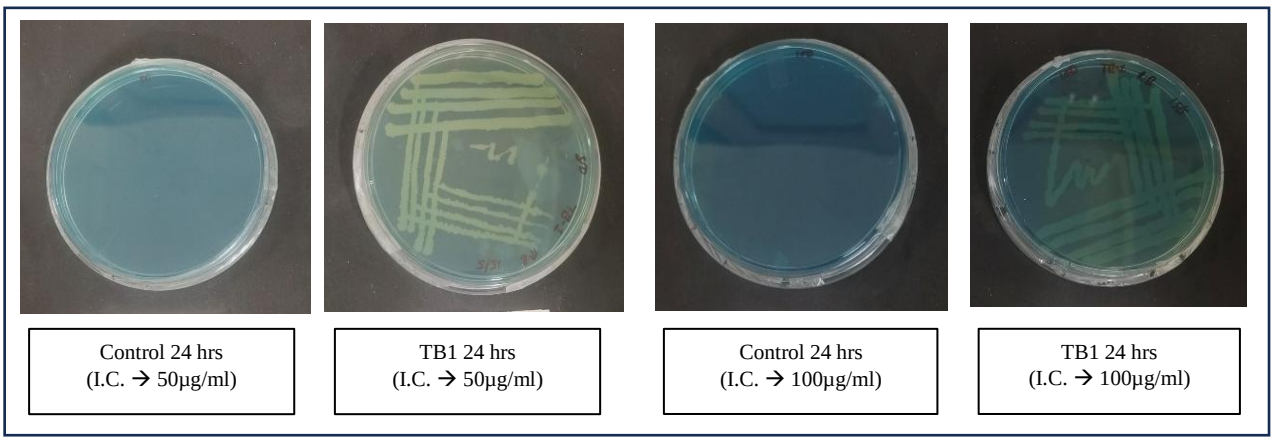
The successfully revived bacterial strains on the solid agar media plates were further identified by performing the 16s rRNA sequencing. The data collected from the chromatogram files showed many bacterial strains whose characteristics matched with the unknown TB1 and TB2 bacterial species. The contiguous sequences were prepared in the PRABI-Doua (CAP3 Sequence Assembly Program). The forward and reverse primer sequences aligned together by removing the gaps are known as contiguous files. Then the fasta files were obtained from the contiguous sequences and were exposed to the Nucleotide Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) database. Finally, the phylogenetic tree was prepared by using the MEGA11 software application. Each of the five selected bacterial species were downloaded as fasta files from NCBI.

Here, I have worked with two bacterial strains, TB1 and TB2 and the phylogenetic tree for the strains were obtained respectively.

5.4) Degradation of Indigo Carmine dye in the solid agar media plates after 24 and 48 hours

Agar media plates were prepared with different concentrations of the Indigo Carmine dye for both the TB1 and TB2 bacterial strains. There were 2 sets, each having 4 plates. In the first set the 4 control plates, individually had the dye concentration of 50µg/ml, 100µg/ml, 200µg/ml and 300µg/ml. In another set there were the rest of the 4 plates that had 4 different concentrations of dye with the respective bacterial strain streaked upon using quadrant streaking method. The solid agar media plates with different Indigo Carmine dye concentration and the bacterial strains (TB1 and TB2) showed the variable dye degradation capacity based on their metabolic function and different cellular morphology as discussed below.

<u>Dye degradation showed by TB1</u>



Control 24 hrs (I.C. → 50µg/ml) | TB1 24 hrs (I.C. → 50µg/ml) | Control 24 hrs (I.C. → 100µg/ml) | TB1 24 hrs (I.C. → 100µg/ml)

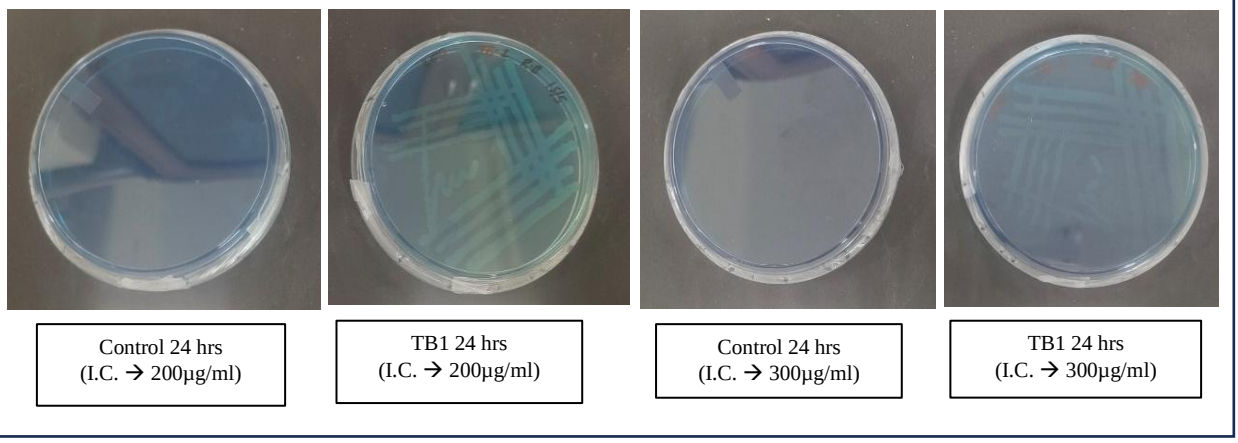
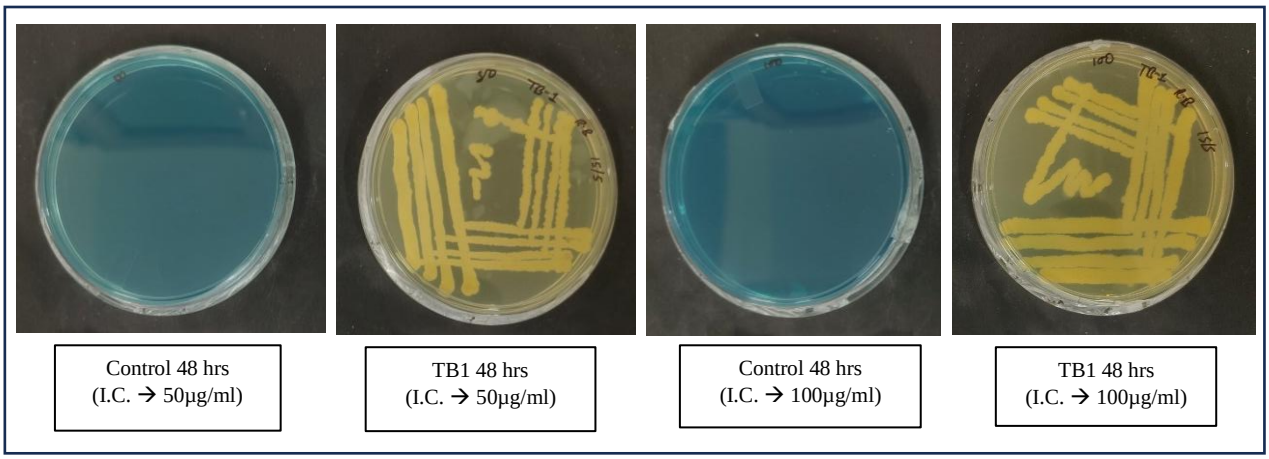


Figure 4: Dye degradation by TB1 in solid agar media plates supplemented with I.C. dye concentrations (50-300 µg/ml) after 24 hours

As the bacterial strain TB1 was isolated and purified, the strain was kept in glycerol stock solution at -80º C. From the stock solution the TB1 strain was streaked on the solidified 4 agar media plates, having the 4 different concentrations of dye (50µg/ml, 100µg/ml, 200µg/ml and 300µg/ml). The plates were kept inside the incubator at 37º C for 24 hours. The next day all the 4 streaked plates respectively with their control were observed for the dye degradation phenomenon. TB1 strain showed circular colony form, raised elevation, entire margin and the colony colour was white. It was observed that each of the streaked plates starting from lower 50 µg/ml dye concentration to higher concentration of 300 µg/ml has degraded the dye. The plate having the lowest concentration of 50 µg/ml and the TB1 strain streak showed the maximum dye degradation. The dye degradation rate was observed to be inversely proportional to the dye concentration because as the concentration kept on increasing the dye degradation rate kept on decreasing and was least at 300 µg/ml.



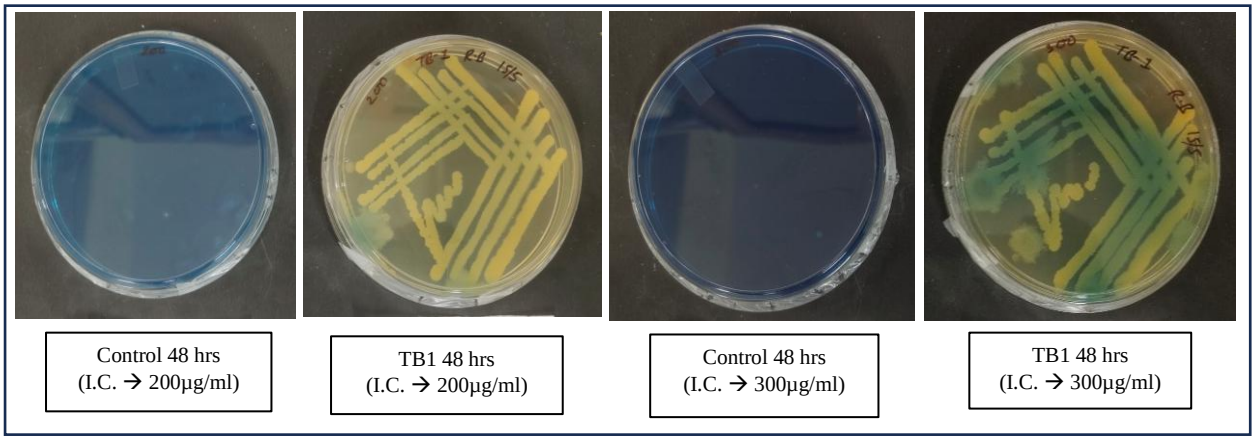


Figure 5: Dye degradation by TB1 in solid agar media plates supplemented with I.C. dye concentrations (50-300 µg/ml) after 48 hours

After the observation, all the 4 plates with dye and TB1 strain with their respected control plates were incubated for more 24 hours that resulted in a total of 48 hours of observation the next day. It was clearly seen that the plate with dye concentration of 50 µg/ml with TB1 strain degraded the dye totally as the colour of the agar plate changed to yellow from blue that proved the total dye degradation after 48 hours of incubation. Similar result was seen for the plate with 100 µg/ml of dye concentration with TB1 streaked. Plates with 200 µg/ml and 300 µg/ml showed inverse relationship with dye degradation as the concentration increased the degradation rate decreased and was least observed with the 300 µg/ml dye concentrated streak plate.

Dye degradation showed by TB2

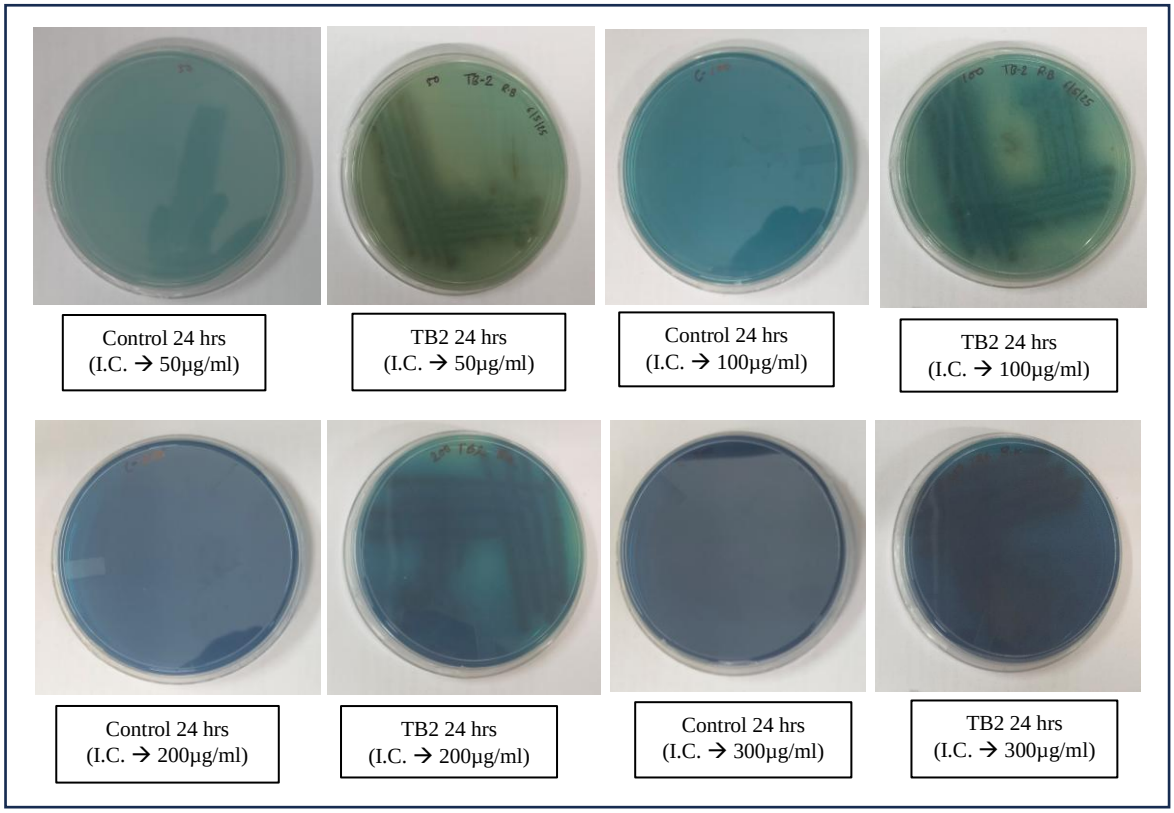


Figure 6: Dye degradation by TB2 in solid agar media plates supplemented with I.C. dye concentrations (50-300 µg/ml) after 24 hours

In this case also 4 plates were there that had 4 different dye concentrations, 50µg/ml, 100µg/ml, 200µg/ml and 300µg/ml. Previously isolated and purified bacterial strain TB2 had glycerol stock solution at -80ºC, which was streaked on all the plates and were incubated for 24 hours at 37º C for the dye degradation observation. Next day as 24 hours completed, the TB2 growth was observed in the streaked plates. It showed white colour colony with regular colony form, raised elevation and lobate margin. TB2 strain also showed clear vesicular structures formation that were assumed to accumulate the dye and degrade it slowly compared to TB1 as it didn't show any such vesicular structure, nor did they accumulate the dye. After observing each plate the next day, it was seen that the plate with 50 µg/ml dye concentration showed higher dye degradation rate than the other 3 concentrations. Here, also as the dye concentration in the agar plates increased the dye degrading capacity of the TB2 decreased that showed an inverse relationship between them. The TB2 strain was observed to accumulate the dye

and degrade it slowly as the streaked lines that showed TB2 growth were bluish in colour besides the colony's prominent white colour. This qualitatively showed that the strain can particularly accumulate the I.C. dye on the whole plate.

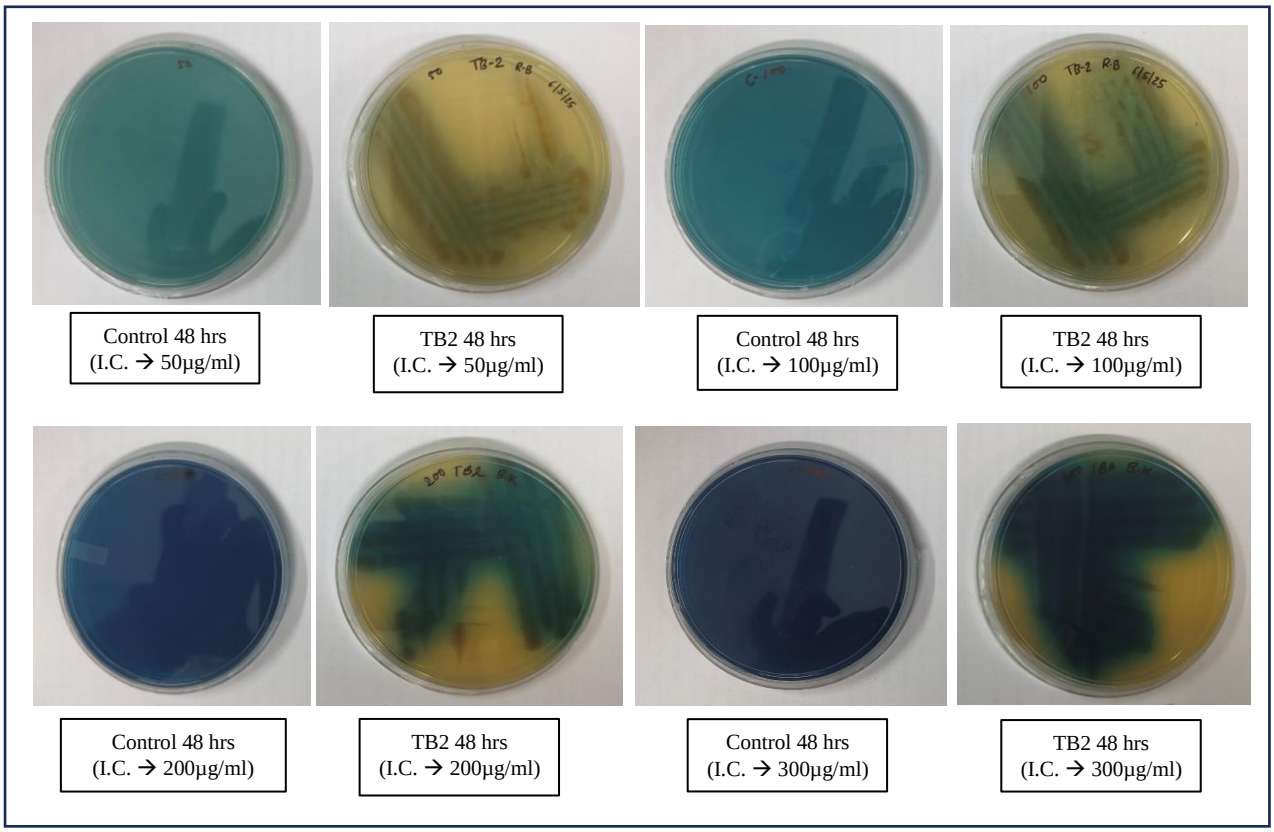


Figure 7: Dye degradation by TB2 in solid agar media plates supplemented with I.C. dye concentrations (50-300 µg/ml) after 48 hours

Again, all the plates were kept for incubation for 48 hours at 37ºC. Next day in total 48hours of observation was to be taken and the plate with least dye concentration of 50 µg/ml showed total dye degradation. Plates with 100µg/ml, 200µg/ml and 300µg/ml dye concentration showed similar results. The dye degradation rate was more than it was observed in 24 hours observation but as the plate with highest dye concentration of 300 µg/ml showed least dye degradation capacity.

5.5) Degradation of Indigo Carmine dye by bacterial strains in the Luria Broth (L.B.) culture tubes in static conditions

Among the total 8 culture tubes, there were 2 sets with 4 tubes in each set. One set contained 10ml of L.B individually with different dye concentration of 50 µg/ml, 100µg/ml, 200µg/ml and 300µg/ml which were controls respectively for another set of

4 culture tubes with different dye concentrations and the inoculated bacterial strains (TB1 and TB2). Observations were taken for 24 hours and 48 hours of incubation at 37º C in static condition, followed by centrifugation and measurement of optical density (O.D.) for both the bacterial strains. The O.D. values were diverse due to the variable dye degradation capacity of the strains & presence of degraded and undegraded dye portions in the L.B. broth with the strains inoculated in each of the different dye concentrations as discussed below.

Dye degradation showed by TB1

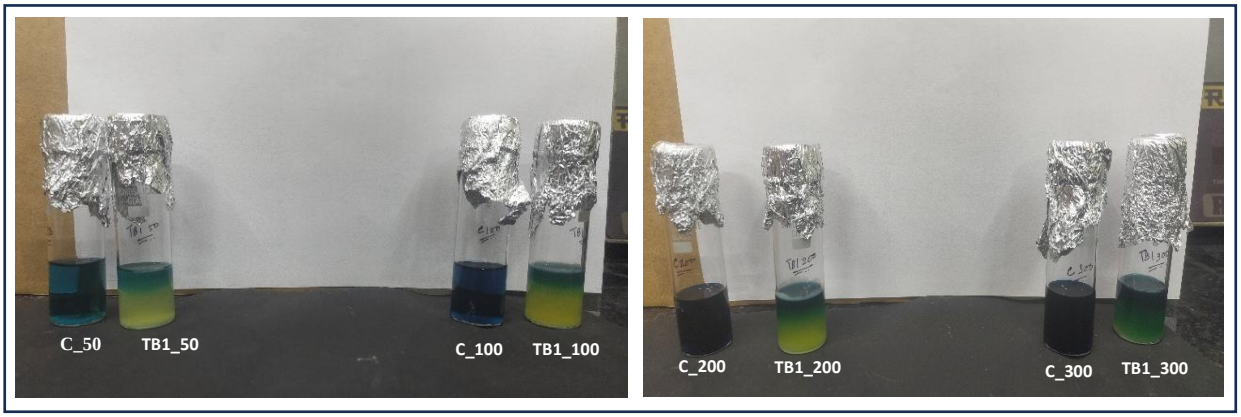


Figure 8: Dye degradation by TB1 in LB+I.C. dye concentrations (50-300 µg/ml) in static condition after 24 hours

After inoculating 4 L.B. culture tubes with loopful of TB1 strain, they were incubated for 24 hours at static conditions in the incubator at 37º C along with control set of 4 L.B. culture tubes. After 24 hours, it was observed that the tube with the least concentration, 50 µg/ml, showed the highest dye degradation capacity. The degraded part was bright yellow in colour in the middle and the undegraded part was dark blue at the bottom. Due to the static condition in incubator, it was also observed that TB1 is capable to form film-like bacterial growth on the surface of the LB + I.C. dye broth media. The tube with 100µg/ml, 200µg/ml and 300µg/ml also showed the dye degradation capacity in a decreased manner that means the tube containing 300µg/ml of dye showed the least degradation rate. The tube with 100µg/ml dye concentration showed the degraded part in yellowish green colour in the middle with dark blue at the bottom which meant the degradation took place at a slower rate compared to the 50 µg/ml. The other 2 tubes with 200 µg/ml and 300 µg/ml dye concentration showed the degraded part in bluish-green color in the middle and dark blue indigo at the bottom of

the culture tube. After the observation 1 ml dye degraded part from each of the 8 culture tubes were extracted out using 1 ml syringe and added into centrifuge tubes and were centrifuged at 5000 revolutions per minute (rpm) for 10 minutes in a spin win. The supernatant was then placed in 96 microtiters well plate reader with half dilution (50µl sample + 50µl d.H_2O) and the O.D was measured at 595 nm. The O.D values were highest for 50 µg/ml and lowest for 300 µg/ml. This showed that in 50 µg/ml tube the dye degradation rate was higher due to the low dye concentration that made it easier for the TB1 strain to degrade the dye quicker than 100 µg/ml, 200 µg/ml and 300 µg/ml as in these tubes the dye concentration was higher. The 50 µg/ml dye concentrated culture tubes inoculated with TB1 had more undegraded dye parts that allowed more light to get transmitted from the sample compared to other 3 dye concentrated culture tubes having 100 µg/ml, 200 µg/ml and 300 µg/ml dye concentration.

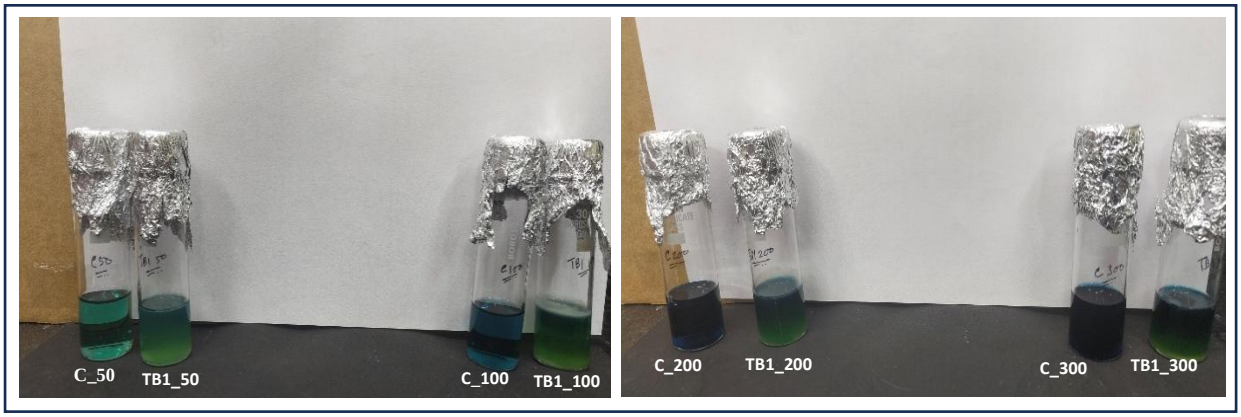


Figure 9: Dye degradation by TB1 in LB+I.C. dye concentrations (50-300 µg/ml) in static condition after 48 hours

As, after 24 hours of observation it was proved that TB1 can degrade the Indigo Carmine dye and to observe more degradation all the inoculated culture tubes with their respective control set were again kept in incubation in static conditions at 37º C for another 24 hours that in whole calculated to be 48 hours observation. After 48 hours, the tube with 50 µg/ml dye concentration totally degraded the dye showing yellow colour of the L.B broth. Similarly, 100µg/ml, 200µg/ml and 300µg/ml dye concentrations showed higher degradation rate compared to 24 hours observation. This time the degraded parts in these tubes were yellow in colour in the middle with very little bluish green colour at the bottom. This showed that even with different dye concentrations, bacterial strain TB1 can degrade the dye successfully. After this the

degraded dye part from all 8 tubes were extracted using 1 ml syringe and added into centrifuge tubes, followed by centrifugation at 5000 rpm for 10 minutes in a spin win. The supernatant was collected, were half-diluted in the 96 microtiters well plate reader and O.D. was measured respectively at 595 nm. This time the O.D. showed drastic high O.D. values for 50 µg/ml dye concentration as the time was more and TB1 strain totally degraded the dye while low O.D values were measured for 300 µg/ml dye concentration. This also showed that the least dye concentration tube had more undegraded dye parts that allowed more light to pass through the sample compared to the all the 3 other dye concentrated culture tubes inoculated with TB1 strain.

	C 50	TB1 50	C 100	TB1 100	C 200	TB1 200	C 300	TB1 300
24 hrs	0.415	0.319	0.494	0.357	1.214	0.556	1.679	0.739
48 hrs	0.426	0.334	0.48	0.389	1.197	0.584	1.712	0.747

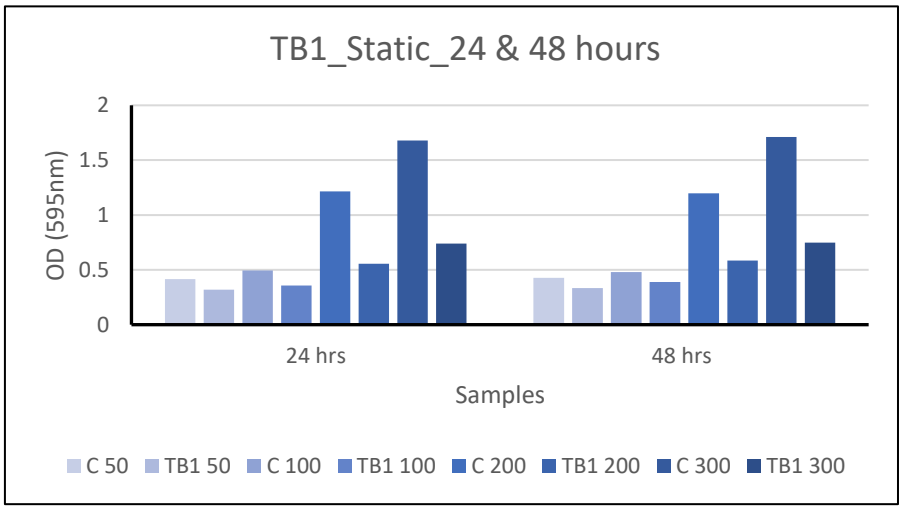


Table 2: Optical density (O.D) table and graphical representation of TB1 strain in LB+I.C. dye concentrations broth media at static condition after 24- and 48-hours showing dye degradation

Dye degradation showed by TB2

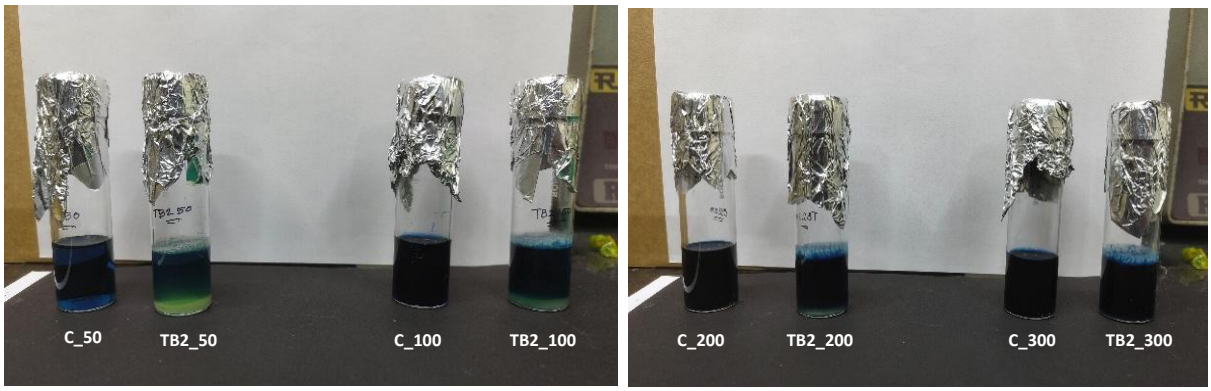


Figure 10: Dye degradation by TB2 in LB+I.C. dye concentrations (50-300 µg/ml) in static condition after 24 hours

8 culture tubes had L.B broth, where 4 tubes had different dye concentrations (50 µg/ml, 100µg/ml, 200µg/ml and 300µg/ml) inoculated with TB2 strain and another 4 were control tubes only L.B. with dye. The culture tubes were incubated for 24 hours of observation at 37º C. After 24 hours, each culture tube showed dye degradation but at different rates. The highest dye degradation rate was observed with 50 µg/ml of dye concentrated and TB2 inoculated tube as the yellow part in the middle showed degraded dye and the blue part at the bottom showed undegraded part. Due to the static conditions the TB2 strain also formed the film like bacterial growth that was clearly visible. Then very carefully 1ml of degraded dye part from each sample was extracted out without disturbing the film formed on the surface of the broth, into the centrifuge tubes. Centrifugation was done in spin win at 5000 rpm for 10 minutes. Then the supernatant was collected in the 96 microtiters well plate reader and after half dilution with distilled water (d.H_2O) they were subjected to measure the O.D at 595 nm. The high O.D values for the lowest dye concentration 50 µg/ml proved that the TB2 strain got more time to degrade the lowest dye concentration therefore, due to the presence of more undegraded dye parts the light transmission was high. While as the dye concentration increased (100µg/ml, 200µg/ml and 300µg/ml) the O.D values decreased showing that the higher concentrations had more undegraded dye parts that didn't allow the light to transmit and so the O.D. values were gradually lower as the dye concentration increased. So, to see the full dye degradation 48 hours observation was also performed.

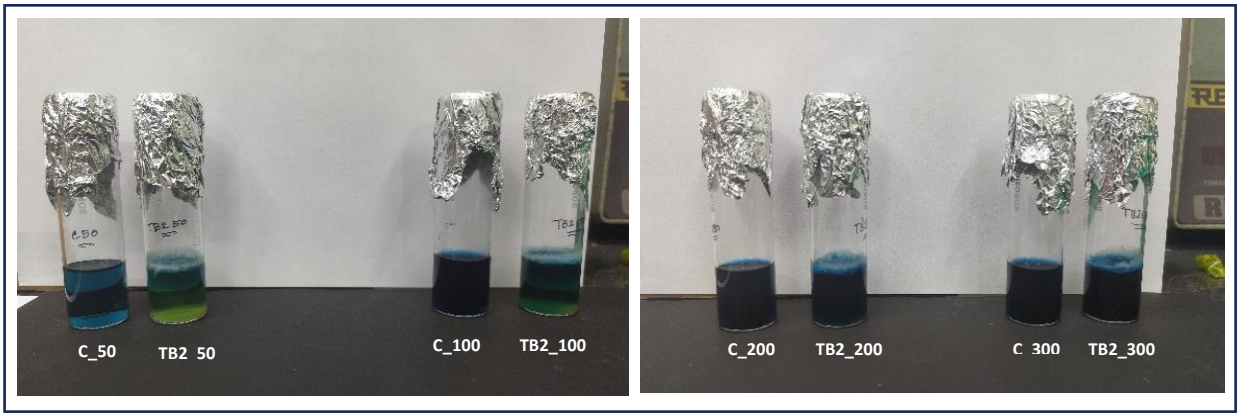


Figure 11: Dye degradation by TB2 in LB+I.C. dye concentrations (50-300 µg/ml) in static condition after 48 hours

After this, again for another 24 hours that made 48 hours of observation the next day, all the 8 culture tubes were incubated at 37ºC, at static conditions. It was observed after 48 hours that each of the 4 different dye concentrated tubes with TB2 strain showed the degradation rate higher than it showed after 24 hours The TB2 inoculated tube with the least dye concentration, 50 µg/ml showed the highest dye degradation capacity because the colour of the L.B. turned totally yellow, showing majorly the dye degraded part which had no indigo colour. Similarly, the rest of the 3 dye concentrations inoculated with TB2 also showed degradation rate higher than 24 hours. The dye degradation capacity decreased with increasing concentration of dye. This was visible because by observing the inoculated culture tubes well enough yellow degraded part in the middle with lower amount of blue undegraded part at the bottom was seen. Films like bacterial growth were also formed in all the 4 inoculated culture tubes due to the incubation at static condition. Again, 1ml of the degraded dye part was extracted out using 1 ml syringe and added into the centrifuge tubes followed by centrifugation at 5000 rpm for 10 minutes in a spin win. The supernatant collected was then half diluted with distilled water and were pipetted out in the 96 microtiters well plate reader to measure the O.D at 595 nm. The least dye concentration showed the highest O.D value that proved it had maximum degraded dye parts that allowed more light to pass through the sample. The O.D values kept gradually decreasing with increasing dye concentration to 300 µg/ml that showed, they also contained undegraded dye that stopped the light to transmit that resulted in decreased O.D values.

	C 50	TB2 50	C 100	TB2 100	C 200	TB2 200	C 300	TB2 300
24 hrs	0.513	0.389	0.579	0.512	1.346	1.014	1.895	1.643
48 hrs	0.485	0.354	0.536	0.475	1.245	0.967	1.736	1.395

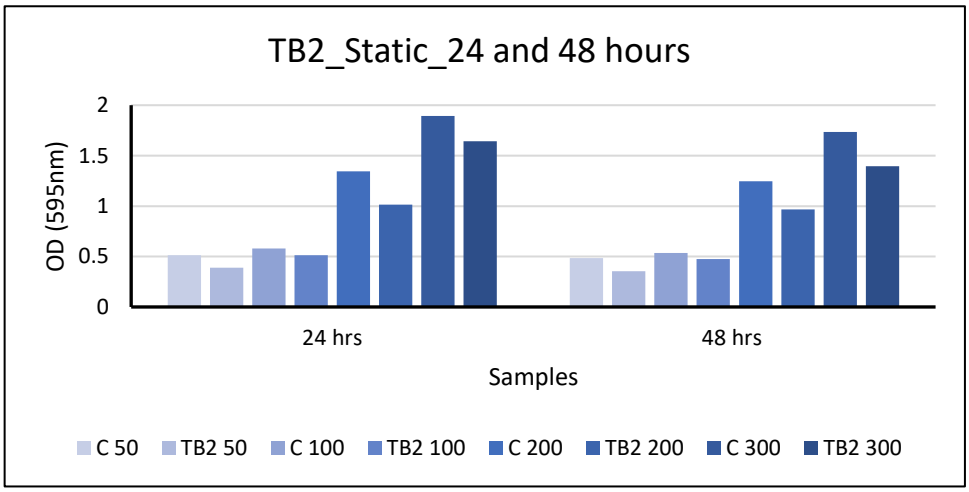


Table 3: Optical density (O.D) table and graphical representation of TB2 strain in LB+I.C. dye concentrations broth media at static condition after 24- and 48-hours showing dye degradation

5.6) Degradation of Indigo Carmine dye by bacterial strains in the Luria Broth (L.B.) culture tubes in shaking conditions at 160 rpm

As the bacterial strains TB1 and TB2 were subjected to the L.B. dye degradation analysis in static condition, similarly under shaking conditions, also the dye degradation analysis was performed. The strains were inoculated and incubated for 24 and 48 hours and each time the dye degradation rate was observed followed by centrifugation process and then O.D. measurement, using the 96 microtiters well plate reader. L.B. was prepared for 8 culture tubes, each containing 10ml of broth. 4 culture tubes were pipetted with 4 different dye concentrations and inoculated with TB1 & TB2 strains. The other 4 culture tubes only contained the different dye concentrated broth. All the tubes were incubated at 37^{o} C for 24 hours at shaking conditions, 160 rpm followed by 48 hours, that gave the result of dye degradation. Centrifugation of all the 8 samples was performed in a spin win apparatus, and the supernatant was half-diluted to measure

the O.D. at 595 nm. The I.C. dye degradation rate varied for the samples with different dye concentration but the same amount of TB1 & TB2 strain that is discussed below.

Dye degradation showed by TB1

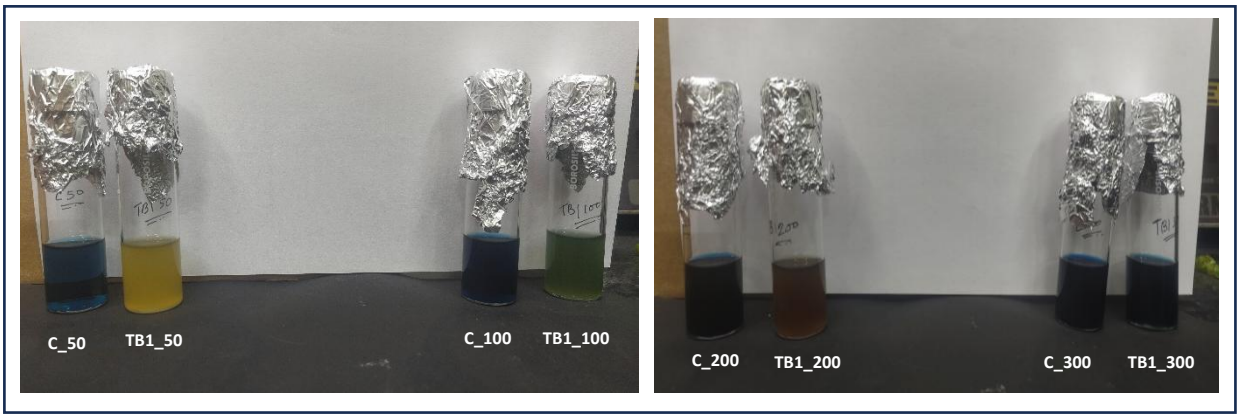


Figure 12: Dye degradation by TB1 in LB+I.C. dye concentrations (50-300 µg/ml) in shaking condition, at 160rpm after 24 hours

Firstly, TB1 strain was inoculated in 4 culture tubes containing 4 variable dye concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml) respectively. With their control tubes all the 8 tubes were incubated at 37º C, at shaking condition of 160 rpm for 24 hours. The next day when the tubes were taken out of the incubator then all the 4 tubes with dye and TB1 strain showed the dye degradation phenomenon but the least concentration, 50 µg/ml showed the highest dye degradation as the maximum middle part of the broth contained more degraded dye parts as the colour changed to yellow indicating degraded dye and lesser undegraded dye portion indicated by lesser amount of dark blue colour at the bottom of the tube. All the other 3 concentrations, (100 µg/ml, 200 µg/ml and 300 µg/ml) also showed yellow coloured degraded dye parts in the middle layer less than 50 µg/ml and the bottom layer contained undegraded dye portion indicated by the dark blue colour. Due to shaking conditions no film formation was observed over the broth surface. After this 1ml from each of the tubes were added using syringe into the centrifuge tubes and was centrifuged in a spin win at 5000 rpm for 10 minutes. The supernatant was then collected carefully and was half diluted with distilled water (50 µl sample + 50 µl d.H_2O). Lastly the O.D. was measured at 595 nm and showed the tube containing 50 µg/ml dye concentration with TB1 inoculated had highest O.D values because dye degradation was seen to be highest here. Followed by 100, 200 and 300 µg/ml dye concentration the O.D value gradually decreased that

showed an inverse relationship between increasing dye concentration and decreasing O.D values. The decreased O.D values were the result of less light transmission from the sample because it contained less degraded but more undegraded dye portions that prevented the light transmission to an extent.

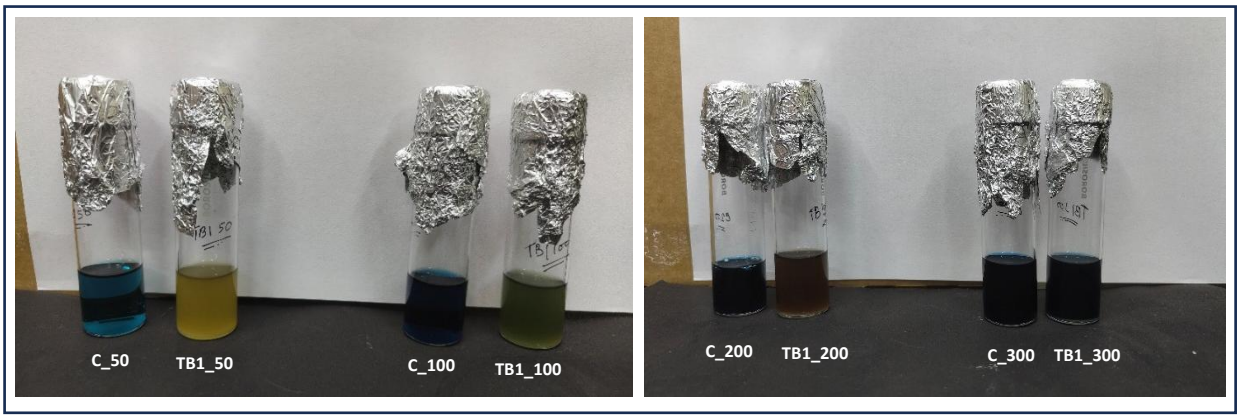


Figure 13: Dye degradation by TB1 in LB+I.C. dye concentrations (50-300 µg/ml) in shaking condition, at 160rpm after 48 hours

After this, all the culture tubes were again very carefully incubated for 48 hours observation at 37º C at 160 rpm shaking conditions to observe more dye degradation. The next day, the similarly least dye concentration containing tube showed the total dye degradation as the tube contained majorly yellow coloured broth indicating the dark blue indigo dye degradation. The other tube with 100 µg/ml dye concentrated TB1 inoculated sample also showed majorly greenish-yellow colour faded broth indicating dye degradation. The other 2 dye concentrated tubes showed a more dye degradation rate compared to 24 hours observation, where faded yellow colour was dominant than the dark blue colour broth. Then 1ml from each of the 8 tubes were added into the centrifuge tubes using syringe. Centrifugation was performed at 5000 rpm for 10 minutes and the supernatant collected was half-diluted with distilled water (50 µl sample + 50 µl d.H_2O) were pipetted into the well plates. O.D was measured at 595 nm using the 96 microtiters well plate reader. The highest O.D values were observed for the least dye concentration, 50 µg/ml as it had more degraded dye portion that allowed more light for transmission. The least O.D value was recorded for the highest dye concentration, 300 µg/ml which indicated the presence of more undegraded dye portion that prevented the light transmission.

	C 50	TB1 50	C 100	TB1 100	C 200	TB1 200	C 300	TB1 300
24 hrs	0.434	0.154	0.563	0.211	1.159	0.638	1.759	0.879
48 hrs	0.47	0.129	0.524	0.194	1.244	0.594	1.684	0.812

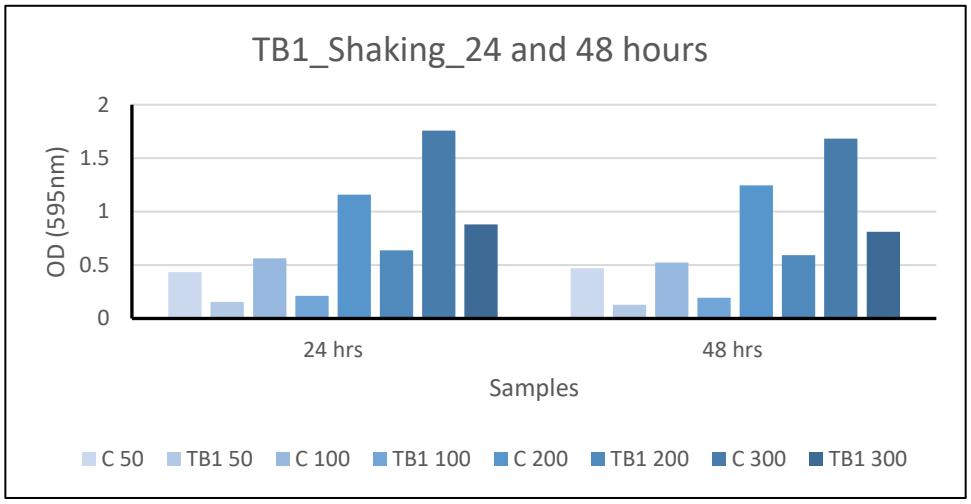


Table 4: Optical density (O.D) table and graphical representation of TB1 strain in LB+I.C. dye concentrations broth media at static condition after 24- and 48-hours showing dye degradation

Dye degradation showed by TB2

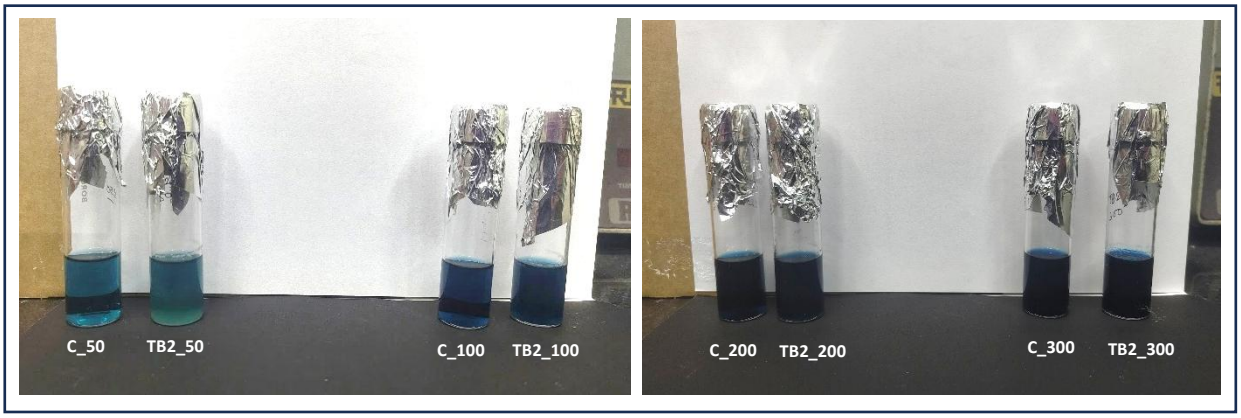


Figure 14: Dye degradation by TB2 in LB+I.C. dye concentrations (50-300 µg/ml) in shaking condition, at 160rpm after 24 hours

All the 8 culture tubes with L.B. was prepared and different dye concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml and 300 µg/ml) was pipetted into them. 4 tubes contained 4 different dye concentrations with TB2 strain inoculated and the rest of the 4 only contained dye as the control. All were kept for incubation at 37º C in shaking conditions in 160 rpm for 24 hours. After 24 hours, when the observation was done, the least dye concentration (50 µg/ml) containing tube showed the highest dye degradation rate as the middle layer was yellow in color indicating the degraded dye portion with less dark blue color at the bottom indicating undegraded dye portion. Other culture tubes with 100 µg/ml, 200 µg/ml and 300 µg/ml of dye concentration also showed the degradation but comparable to 50 µg/ml the degradation rate was slower. In 100 µg/ml, middle layer contained yellowish green color indicating degradation and rest of the two (200 µg/ml and 300 µg/ml) dye concentration showed bluish – green color in the middle layer with dark blue at the bottom layer. Due to shaking condition, the film formation wasn't observed as the bacterial strain didn't get any stagnant condition. Then 1 ml from each sample was added into the respective centrifuge tubes using syringe. Centrifugation was performed at 5000 rpm for 10 minutes and the supernatant was collected out and half-diluted (50 µl sample + 50 µl d.H_2O) in the 96 microtiters well plate reader. After this O.D was measured at 595 nm and the sample with 50 µg/ml of the dye concentration showed the highest O.D value as it had more degraded dye portion than the rest of the 3 concentrations. Similarly, as an inverse relationship, the least O.D was measured for 300 µg/ml dye concentration containing sample of TB2 as there were more undegraded dye portions that prevented a certain extent of light from getting transmitted.

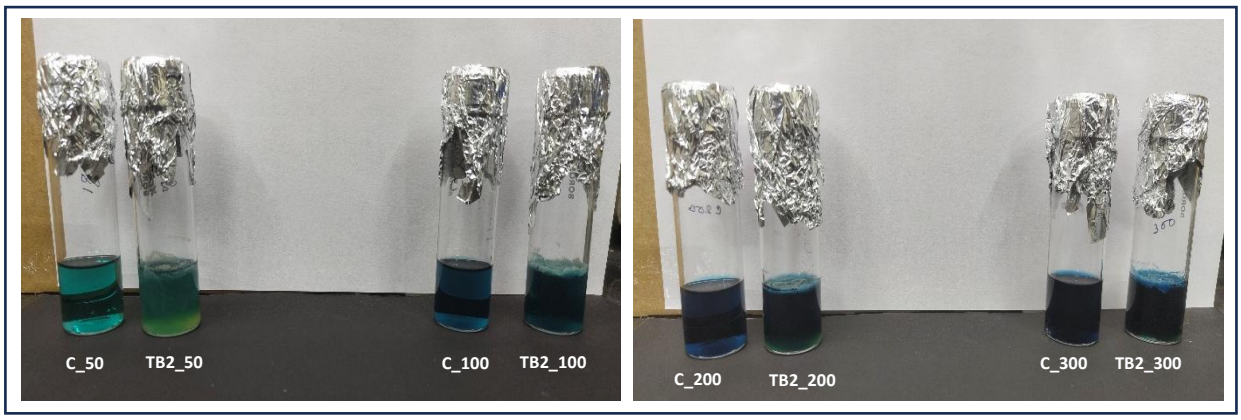


Figure 15: Dye degradation by TB2 in LB+I.C. dye concentrations (50-300 µg/ml) in shaking condition, at 160rpm after 48 hours

To check more dye degradation by the TB2 strain again the 8 culture tubes were kept inside the incubator at the shaking condition of 160 rpm at 37º C for more 24 hours that calculated up to total 48 hours observation. After 48 hours, it was observed that the least concentration of dye containing TB2 inoculated L.B. showed total dye degradation with strong and majorly yellow coloured broth. Similar result was seen for 100 µg/ml, but the bottom layer still contained dark blue undegraded portion but lesser compared to the observation of 24 hours. The other two concentrations also showed now yellowish green colour of the middle-degraded dye portion with a dark blue portion at the bottom indicating the undegraded dye. All the samples were then subjected to centrifugation technique after 1ml from each sample was extracted and added into the centrifuge tubes. Centrifugation was done in the spin win apparatus at 5000 rpm for 10 minutes. The supernatant collected was half-diluted by distilled water (50 µl sample + 50 µl d.H_2O) and were carefully pipetted into the 96 microtiters well plate reader and the O.D was measured at 595 nm. The 50 µg/ml dye concentration inoculated with TB2 strain showed the highest O.D value as it contained majorly the degraded dye portion. The least O.D value was observed for the highest dye concentrated (300 µg/ml) and TB2 inoculated culture tube because it had majorly undegraded dye portion that stopped a certain extent of light from being transmitted from the sample.

	C 50	TB2 50	C 100	TB2 100	C 200	TB2 200	C 300	TB2 300
24 hrs	0.476	0.417	0.586	0.523	1.211	1.091	1.944	1.863
48 hrs	0.439	0.372	0.496	0.482	1.018	0.994	1.874	1.652

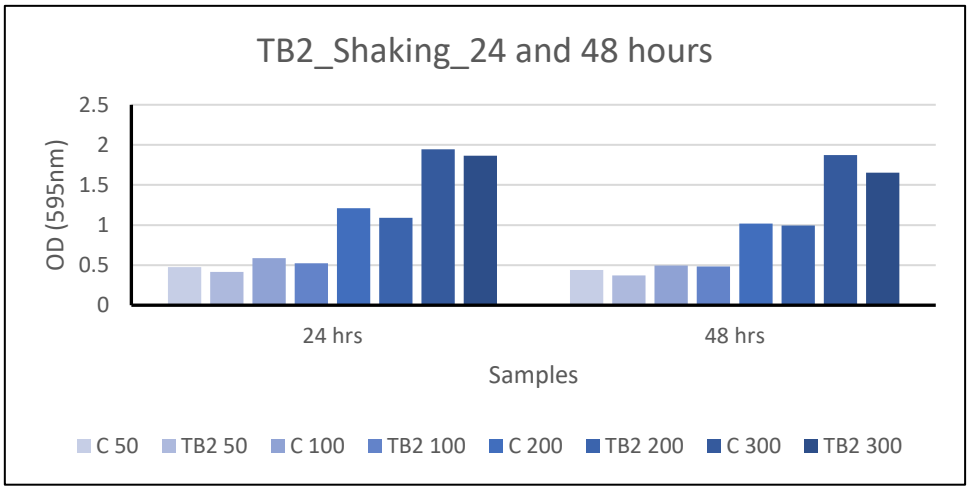


Table 5: Optical density (O.D) table and graphical representation of TB2 strain in LB+I.C. dye concentrations broth media at static condition after 24- and 48-hours showing dye degradation

5.8) Decolorization% calculation to find out the efficient dye-degradation capacity of TB1 and TB2 bacterial strains based on their O.D. values after 24 and 48 hours of dye-degradation analysis in static and 160 rpm shaking conditions

Decolorization% shows the efficient dye-degradation rate by the bacterial species in the form of percentage. Here, decolorization% was calculated for TB1 and TB2 strains respectively inoculated in the L.B. + I.C. dye concentrations liquid broth media.

The calculation of decolorization% was done using the formula:

- Decolorization% = [(Initial absorbance – Final absorbance) / Initial absorbance] *100%

Sl.No.	Sample Name	24 hrs OD	Decolorization% (24 hours_static)	48 hrs OD	Decolorization% (48 hours_static)
1	C-50	0.415	(0.415-0.319/0.415) * 100% = 23.1%	0.426	(0.426-0.334/0.426) * 100 = 21.5%
	TB1-50	0.319		0.334	
2	C-100	0.494	(0.494-0.357/0.494) * 100% = 27.7%	0.48	(0.48-0.389/0.48) * 100 = 18.9%
	TB1-100	0.357		0.389	
3	C-200	1.214	(1.214-0.556/1.214) * 100% = 54.2%	1.197	(1.197-0.584/1.197) * 100 = 51.2%
	TB1-200	0.556		0.584	
4	C-300	1.679	(1.679-0.739/1.679) * 100% = 55.9%	1.712	(1.712-0.747/1.712) * 100 = 56.35%
	TB1-300	0.739		0.747	

Table 6: Decolorization% calculation of TB1 bacterial strain inoculated in LB+I.C. dye concentrations broth media at static condition after 24 and 48 hours

Sl.No.	Sample Name	24 hrs OD	Decolorization% (24 hours_static)	48 hrs OD	Decolorization% (48 hours_static)
1	C-50	0.513	(0.513-0.389/0.513) * 100 = 24.1%	0.485	(0.485-0.354/0.485) * 100 = 27%
	TB2-50	0.389		0.354	
2	C-100	0.579	(0.579-0.512/0.579) * 100 = 11.5%	0.536	(0.536-0.475/0.536) * 100 = 11.3%
	TB2-100	0.512		0.475	
3	C-200	1.346	(1.346-1.014/1.346) * 100 = 24.6%	1.245	(1.245-0.967/1.245) * 100 = 22.3%
	TB2-200	1.014		0.967	
4	C-300	1.895	(1.895-1.643/1.895) * 100 = 13.2%	1.736	(1.736-1.395/1.736) * 100 = 19.6%
	TB2-300	1.643		1.395	

Table 7: Decolorization% calculation of TB2 bacterial strain inoculated in LB+I.C. dye concentrations broth media at static condition after 24 and 48 hours

Sl.No.	Sample Name	24 hrs OD	Decolorization% (24 hours_shaking)	48 hrs OD	Decolorization% (48 hours_shaking)
1	C-50	0.434	(0.434-0.154/0.434) * 100 = 64.5%	0.47	(0.47-0.129/0.47) * 100 = 72.5%
	TB1-50	0.154		0.129	
2	C-100	0.563	(0.563-0.211/0.563) * 100 = 62.5%	0.524	(0.524-0.194/0.524) * 100 = 62.9%
	TB1-100	0.211		0.194	
3	C-200	1.159	(1.159-0.638/1.159) * 100 = 44.9%	1.244	(1.244-0.594/1.244) * 100 = 52.2%
	TB1-200	0.638		0.594	
4	C-300	1.759	(1.759-0.879/1.759) * 100 = 50%	1.684	(1.684-0.812/1.684) * 100 = 51.7%
	TB1-300	0.879		0.812	

Table 8: Decolorization% calculation of TB1 bacterial strain inoculated in LB+I.C. dye concentrations broth media at shaking condition, at 160 rpm after 24 and 48 hours

Sl.No.	Sample Name	24 hrs OD	Decolorization% (24 hours_shaking)	48 hrs OD	Decolorization% (48 hours_shaking)
1	C-50	0.476	(0.476-0.417/0.476) * 100 = 12.3%	0.439	(0.439-0.372/0.439) * 100 = 15.2%
	TB2-50	0.417		0.372	
2	C-100	0.586	(0.586-0.523/0.586) * 100 = 10.75%	0.496	(0.496-0.482/0.496) * 100 = 2%
	TB2-100	0.523		0.482	
3	C-200	1.211	(1.211-1.091/1.211) * 100 = 9.9%	1.018	(1.018-0.994/1.018) * 100 = 2%
	TB2-200	1.091		0.994	
4	C-300	1.944	(1.944-1.863/1.944) * 100 = 4.1%	1.874	(1.874-1.652/1.874) * 100 = 11.8%
	TB2-300	1.863		1.652	

Table 9: Decolorization% calculation of TB2 bacterial strain inoculated in LB+I.C. dye concentrations broth media at shaking condition, at 160 rpm after 24 and 48 hours

The decolorization% was calculated for each experimental set-up with respect to each of their respective control set-up for both the TB1 and TB2 bacterial species. The decolorization% of TB1-50 has been calculated with respect to the O.D. measured in its control set-up, C-50. In this way the decolorization% of TB1 and TB2 with different dye concentrations with respect to their control set-up were calculated quantitatively. From this calculation it was significantly evident that the decolorization was obtained at every step with each of the 4 different I.C. dye supplemented L.B. liquid media. The effective decolorization% by TB1 and TB2 was observed to be better in the shaking set-up compared to the static set-up.

6. Conclusion

The present study successfully demonstrates the potential of indigenous bacterial species isolated from textile effluents in the degradation of synthetic dyes, offering a sustainable alternative to conventional physicochemical treatment methods. Through systematic isolation, screening, and identification, several efficient dye-degrading bacterial strains were identified, primarily belonging to the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Aeromonas*. These bacteria exhibited notable dye decolorization abilities, especially under optimized laboratory conditions, indicating their adaptability and robustness in dye-polluted environments. Furthermore, the study highlighted the influence of key environmental parameters—such as pH, temperature, and dye concentration—on the efficiency of dye degradation. Optimal conditions significantly enhanced the degradation performance of the isolates, suggesting that controlled bioreactor systems could maximize their bioremediation potential. The adaptability of these bacteria to varying environmental conditions also points to their viability in real-world effluent treatment applications. Importantly, the isolation of these bacteria from textile effluents themselves implies that they are naturally acclimated to the toxic and variable conditions of such wastewater. This native resilience enhances their suitability for in situ applications, reducing the need for extensive acclimatization or genetic modification. The ability of some strains to tolerate and degrade multiple types of dyes also suggests the feasibility of using bacterial consortia to achieve broad-spectrum dye degradation. In conclusion, this study provides a strong foundation for the use of dye-degrading bacteria as an eco-friendly and cost-effective approach to mitigate the environmental impact of textile industry effluents. The identified bacterial species hold promise for further development into bioremediation agents for wastewater treatment plants. Future work should focus on scaling up the application through bioreactor design, microbial consortium development, and field trials to validate their effectiveness in diverse and dynamic industrial settings. Integrating such biological treatment methods could significantly enhance the sustainability of wastewater management in the textile sector.

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